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RESEARCH PAPER

The roles and mechanisms of PAR4 and $P2Y_{12}$/phosphatidylinositol 3-kinase pathway in maintaining thrombin-induced platelet aggregation

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BACKGROUND AND PURPOSE
Activation of human platelets by thrombin is mediated predominately through two proteinase-activated receptors (PARs), PAR1 and PAR4. Phosphatidylinositol 3-kinase (PI3K) inhibition leads to reversible PAR1-mediated platelet aggregation, but has no effect on the stability of platelet aggregation induced by thrombin. In the present study, the molecular mechanisms underlying this difference were investigated.

EXPERIMENTAL APPROACH
The functions of PI3K and PAR4 were assessed using specific inhibitors and aggregometry. The duration of platelet glycoprotein (GP) IIb/IIIa exposure was determined by flow cytometry with the antibody PAC-1. Western blotting and fluo-3 was used to evaluate the activation of Akt and protein kinase C (PKC) and intracellular Ca^{2+} mobilization respectively.

KEY RESULTS
When PAR4 function was inhibited either by the PAR4 antagonist YD-3 [1-benzyl-3-(ethoxycarbonylphenyl)-indazole] or by receptor desensitization, the PI3K inhibitor wortmannin turned thrombin-elicited platelet aggregation from an irreversible event to a reversible event. Moreover, wortmannin plus YD-3 markedly accelerated the inactivation of GPIIb/IIIa in thrombin-stimulated platelets. The aggregation-reversing activity mainly resulted from inhibition of both PI3K-dependent PKC activation and PAR4-mediated sustained intracellular Ca^{2+} rises. Blockade of ADP $P2Y_{12}$ receptor with 2-methylthioadenosine 5′-monophosphate triethylammonium salt mimicked the inhibitory effect of wortmannin on PI3K-dependent PKC activation and its ability to reverse PAR1-activating peptide-induced platelet aggregation. Co-administration of 2-methylthioadenosine 5′-monophosphate triethylammonium salt with YD-3 also decreased the stability of thrombin-induced platelet aggregation.

CONCLUSIONS AND IMPLICATIONS
These results suggest that PAR4 acts in parallel with the $P2Y_{12}$/PI3K pathway to stabilize platelet aggregates, and provide new insights into the mechanisms of thrombus stabilization and potential applications for antithrombotic therapy.

Abbreviations
2Me-SAMP, 2-methylthioadenosine 5′-monophosphate triethylammonium salt; AP, activating peptide; GPIIb/IIIa, glycoprotein IIb/IIIa; MARCKS, myristoylated alanine-rich C kinase substrate; PAR, proteinase-activated receptor; PDK-1, phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; TPA, 12-O-tetradecanoylphorbol 13-acetate

Introduction

Thrombin is a serine protease playing a central role in both haemostasis and thrombosis (Crawley *et al.*, 2007; Martorell *et al.*, 2008). In the blood coagulation cascade, thrombin is the final key enzyme, cleaving fibrinogen to form fibrin. Moreover, thrombin is also the most potent platelet activator. Activation of human platelets by thrombin is mediated predominately through two proteinase-activated receptors (PARs), PAR1 and PAR4 (PAR_1 and PAR_4), which belong to the G protein-coupled receptor family (Coughlin, 2005; Leger *et al.*, 2006; Alexander *et al.*, 2009). Both PAR1 and PAR4 couple to phospholipase Cβ (PLCβ) via G_q in human platelets. Upon activation, PLCβ hydrolyses phosphatidylinositol 4,5-bisphosphate to inositol-3-phosphate, which contributes to calcium release from internal stores, and diacylglycerol (DAG), which activates protein kinase C (PKC). PAR1 and PAR4 also couple to $G_{12}/_{13}$ to activate Rho/Rho kinase (Woulfe, 2005). The G_q/PLCβ pathway is essential for glycoprotein IIb/IIIa (GPIIb/IIIa) activation and platelet aggregation, while the $G_{12/13}$/Rho pathway is involved in platelet shape change (Offermanns, 2006). Whether PAR1 and PAR4 directly couple to G_i is still controversial; however, they are apparently able to activate the G_i pathway indirectly through released ADP binding to $P2Y_{12}$ receptor (Kim *et al.*, 2004; Resendiz *et al.*, 2007; Voss *et al.*, 2007). Stimulation of the G_i pathway leads to either inhibition of adenylate cyclase or activation of phosphatidylinositol 3-kinase (PI3K).

Although PAR1 and PAR4 seem to couple to the same set of heterotrimeric G proteins and signalling molecules in human platelets, their signals differ in the timing and magnitude. It is known that PAR1 triggers a rapid and transient increase in intracellular calcium while PAR4 triggers a slower but more prolonged response (Shapiro *et al.*, 2000; Covic *et al.*, 2002b). The differences in the kinetics of the signals mediated by PAR1 and PAR4 imply that the two PARs may play distinct roles in the early and late events of platelet activation. For example, it has been suggested that PAR1 accounts for the initial platelet aggregation in response to thrombin, while PAR4 may contribute to the stability of platelet aggregation (Covic *et al.*, 2002b). In our previous studies, a synthetic benzyl indazole derivative YD-3 [1-benzyl-3-(ethoxycarbonylphenyl)-indazole] was found to be a potent and selective non-peptide PAR4 antagonist in human and mouse platelets (Wu *et al.*, 2002). By using YD-3 as a research tool, we demonstrated that PAR4-mediated prolonged calcium signal is important for sustained phospholipase A_2 activation and thromboxane formation in thrombin-stimulated human platelets (Wu *et al.*, 2003).

Inhibition of PI3K by wortmannin has been found to reverse platelet aggregation and inhibit the maintenance of GPIIb/IIIa activation in response to PAR1-activating peptide (PAR1-AP), suggesting that PI3K plays a critical role in maintaining irreversible platelet aggregation (Kovacsovics *et al.*, 1995). However, wortmannin does not affect the stability of the platelet aggregation induced by thrombin or PAR4-activating peptide (PAR4-AP) (Voss *et al.*, 2007). The mechanisms underlying this difference, particularly the intracellular signalling pathway, still remain to be fully elucidated. In the present study, we investigated the roles and mechanisms of PI3K and PAR4 in the irreversible platelet aggregation caused by thrombin. Our results demonstrate that PAR4 and PI3K act in parallel to maintain thrombin-induced GPIIb/IIIa activation and platelet aggregation. Moreover, the irreversible platelet aggregation induced by PI3K and PAR4 is mediated through prolonged PKC activation and an increase in intracellular Ca^{2+}.

Methods

Preparation of washed human platelets

Human blood anticoagulated with acid citrate dextrose was obtained from healthy human volunteers who had not taken any drugs within the last 2 weeks. The platelet suspension was then prepared according to the washing procedure described previously (Wang *et al.*, 2006). Platelets were finally suspended in Tyrode's solution containing Ca^{2+} (2 mM), glucose (11.1 mM) and bovine serum albumin (3.5 $mg{\cdot}mL^{-1}$) at a concentration of 3×10^8 $platelets{\cdot}mL^{-1}$. For PAR4 desensitization studies, washed platelets were incubated with PAR4-AP (200 μM) at room temperature for 30 min without stirring. To prevent platelet activation during the treatment with PAR4-AP, the platelet inhibitor prostaglandin E_1 (PGE_1, 1 μM) was included in the platelet suspension. After PAR4-AP treatment, the platelets were washed once to remove PGE_1 and PAR4-AP and left to stand for 30 min before testing.

Measurement of platelet aggregation

Platelet aggregation was measured turbidimetrically with a light transmission aggregometer (Chrono-Log Co., Havertown, PA, USA) under a stirring condition (1200 rpm) at 37°C. The extent of platelet aggregation was measured as the maximal increase of light transmission within 5 min after the addition of stimulators. In all experiments, the final concentration of dimethyl sulphoxide (DMSO) was fixed at

0.5% in the samples – a concentration that has no effect on platelet aggregation.

Measurement of PAC-1 binding by flow cytometry

The duration of platelet GPIIb/IIIa exposure was determined by the method described previously (Kovacsovics *et al.*, 1995) using FITC-conjugated PAC-1 monoclonal antibody, which only recognizes the active form of GPIIb/IIIa. Washed human platelets (3×10^7 platelets·mL^{-1}) were pre-incubated with DMSO or test compounds for 5 min; FITC-conjugated PAC-1 was then added either immediately before or 1, 3 or 6 min following thrombin stimulation. Twenty minutes after stimulation, the samples were fixed with 1% paraformaldehyde. Flow cytometric analysis was performed on a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. Platelets were identified by logarithmic signal amplification for forward and side scatter. The levels of PAC-1 binding were expressed as the percentages of cells positive for PAC-1.

Measurement of intracellular Ca^{2+} mobilization

Intracellular Ca^{2+} mobilization of platelets was measured by the method described previously (Wang *et al.*, 2006). In brief, platelets were incubated with fluo-3/AM (3 μM) at 37°C for 30 min. In order to prevent leakage of dye, probenecid (2.5 mM) was added to the buffers throughout the experiments. After washing twice, the fluo-3-loaded platelets were finally suspended in Ca^{2+}-free Tyrode's solution at a concentration of 5×10^7 platelets·mL^{-1}. Calcium (1 mM) was added to the fluo-3-loaded platelets 3 min before stimulation. Fluorescence (Ex 505 nm, Em 530 nm) was measured with a fluorescence spectrophotometer (Model F4000; Hitachi, Tokyo, Japan). Cytosolic free calcium concentration was calculated by the method of Merritt *et al.* (1990).

Platelet lysis and Western blotting

To prepare whole platelet lysates, the reaction was terminated at the indicated time points by addition of 2× SDS sample buffer and boiling for 5 min. Platelet lysates were electrophoresed on an SDS-polyacrylamide gel, and Western blotting was performed as previously described (Wang *et al.*, 2007).

Statistics

Results are expressed as the mean± SEM. Statistical significance was calculated by one-way or two-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Materials

YD-3 was synthesized based on the methods described previously (Chen *et al.*, 2008). Bovine α-thrombin, wortmannin, 2-methylthioadenosine 5′-monophosphate triethylammonium salt (2Me-SAMP), 12-O-tetradecanoylphorbol 13-acetate (TPA), UCN-01 and fluo-3/AM were obtained from Sigma Chemical Co., St. Louis, MO, USA. SCH-79797 was purchased from Tocris, Bristol, UK. PAR1-AP (SFLLRN-NH_2) and PAR4-AP (AYPGKF-NH_2) were purchased from Bachem Biosciences, King of Prussia, PA, USA. FITC-conjugated PAC-1 was purchased from BD Biosciences, San Jose, CA, USA. Phospho-MARCKS (myristoylated alanine-rich C kinase substrate)-specific polyclonal antibody, SH-6 and Akt inhibitor V were purchased from Calbiochem, San Diego, CA, USA. Phospho-Akt (Ser473 and Thr308) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). All other chemicals were purchased from Sigma Chemical Co.

Results

PAR4 is involved in maintaining thrombin-induced platelet aggregation

In washed human platelets, PAR1-AP (20 μM) induced a maximal and sustained platelet aggregation. Pretreatment of platelets with the PI3K inhibitor wortmannin (200 nM) for 5 min before the addition of PAR1-AP led to an initial aggregation, followed by a rapid disaggregation of platelets (Figure 1A). In contrast, wortmannin had little effect on platelet aggregation induced by thrombin (0.1 U·mL^{-1}) or PAR4-AP (100 μM) (Figure 1B and C).

Thromboxane A_2 and ADP are released from activated platelets and act as positive feedback mediators that amplify platelet activation. In order to investigate the contribution of these mediators to wortmannin-resistant platelet aggregation induced by thrombin, specific inhibitors were used. Figure 1D shows that neither the cyclooxygenase inhibitor indomethacin (10 μM) nor the ADP scavenger apyrase (1 U·mL^{-1}) enhanced the effect of wortmannin. Therefore, thromboxane A_2 and ADP are apparently not required for the PI3K-independent irreversible aggregation.

Next, we investigated the role of PAR4 in maintaining thrombin-induced platelet aggregation. As shown in Figure 2A, in the presence of wortmannin, the PAR4 antagonist YD-3 (5, 10 and 20 μM) reduced and/or reversed thrombin (0.1 U·mL^{-1})-induced platelet aggregation in a concentration-

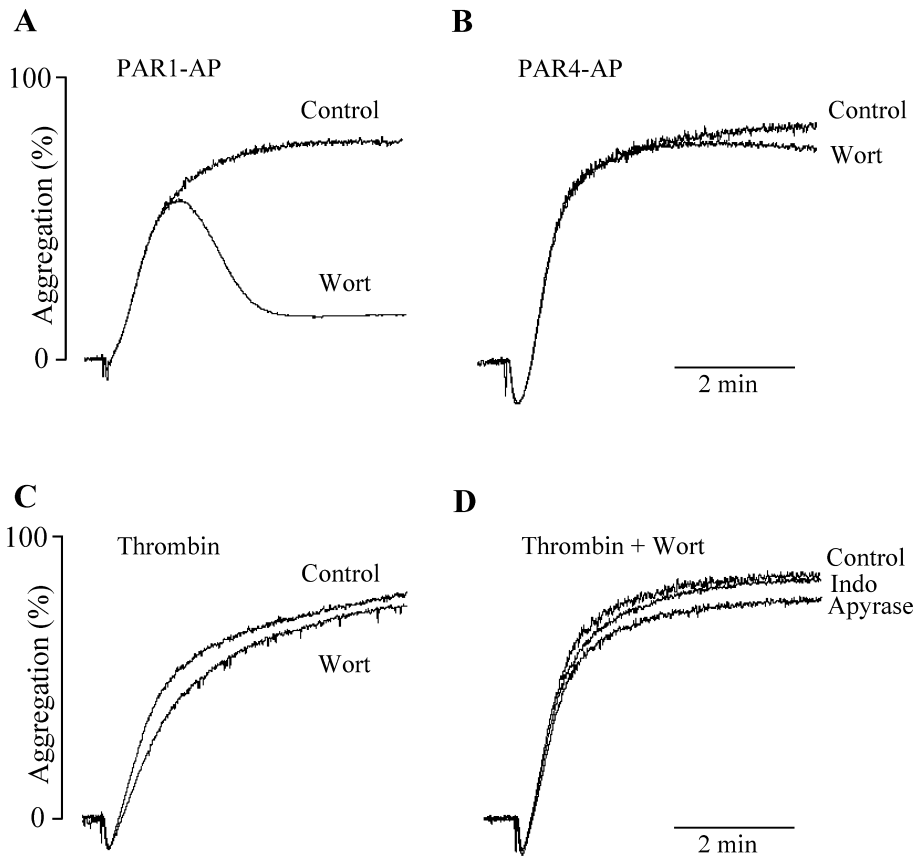

Figure 1

Wortmannin reversed platelet aggregation induced by PAR1-AP, but not that induced by thrombin or PAR4-AP. Washed human platelets were incubated with DMSO (vehicle control) or wortmannin (Wort, 200 nM) at 37°C for 5 min, then 20 μM PAR1-AP (A), 100 μM PAR4-AP (B) or 0.1 U·mL^{-1} thrombin (C) was added to induce platelet aggregation. (D) Platelets were pre-incubated with or without indomethacin (indo, 10 μM) or apyrase (1 U·mL^{-1}) in the presence of wortmannin, and then thrombin was added to induce platelet aggregation. Representative traces of at least four independent experiments are shown. AP, activating peptide; DMSO, dimethyl sulphoxide; PAR, proteinase-activated receptor.

dependent manner; this effect was optimal with 20 μM YD-3. At this concentration, YD-3 completely inhibited thrombin-elicited PAR4 activation (Wu *et al.*, 2002; Wu and Teng, 2006) but only slightly reduced platelet aggregation in response to thrombin on its own. Co-administration of LY294002 (100 μM), another inhibitor of PI3K, and YD-3 also reversed thrombin-induced platelet aggregation (data not shown). Furthermore, post-addition of PAR4-AP to PAR1-stimulated platelets attenuated the inhibitory effect of wortmannin on platelet aggregation (Figure 2B, left panel). Wortmannin was also unable to elicit platelet disaggregation when platelets were simultaneously stimulated with PAR1-AP and PAR4-AP (Figure 2B, right panel). In contrast to YD-3, the PAR1 antagonist SCH-79797 (10 μM), either alone or in combination with wortmannin, did not affect the stability of thrombin-induced platelet aggregation (data not shown).

We tried to confirm further, the role of PAR4 in maintaining irreversible aggregation by the use of PAR4 antagonists other than YD-3. Unfortunately, the PAR4 antagonist, *trans*-cinnamoyl-YPGKF-NH_2 that can block PAR4 signalling in rodent cells (Hollenberg and Saifeddine, 2001; Strande *et al.*, 2008) did not inhibit PAR4-AP-induced aggregation of human platelets (data not shown). Further, the PAR4 pepducin antagonist P4pal-10 (Covic *et al.*, 2002a) was not available to us. Thus, we turned to a receptor desensitization protocol to assess the role of PAR4. Platelets were first treated with PAR4-AP to desensitize PAR4 and were then treated with either PAR1-AP or PAR4-AP to assess their responsiveness. As shown in Figure 2C (left panel), PAR4-desensitized platelets no longer aggregated in response to PAR4-AP, but aggregated irreversibly in response to PAR1-AP, indicating that the desensitization process was specific. In PAR4-desensitized platelets, thrombin induced a smaller but irreversible platelet aggregation, while in the presence of wortmannin, thrombin-induced platelet aggregation became reversible (Figure 2C, right panel).

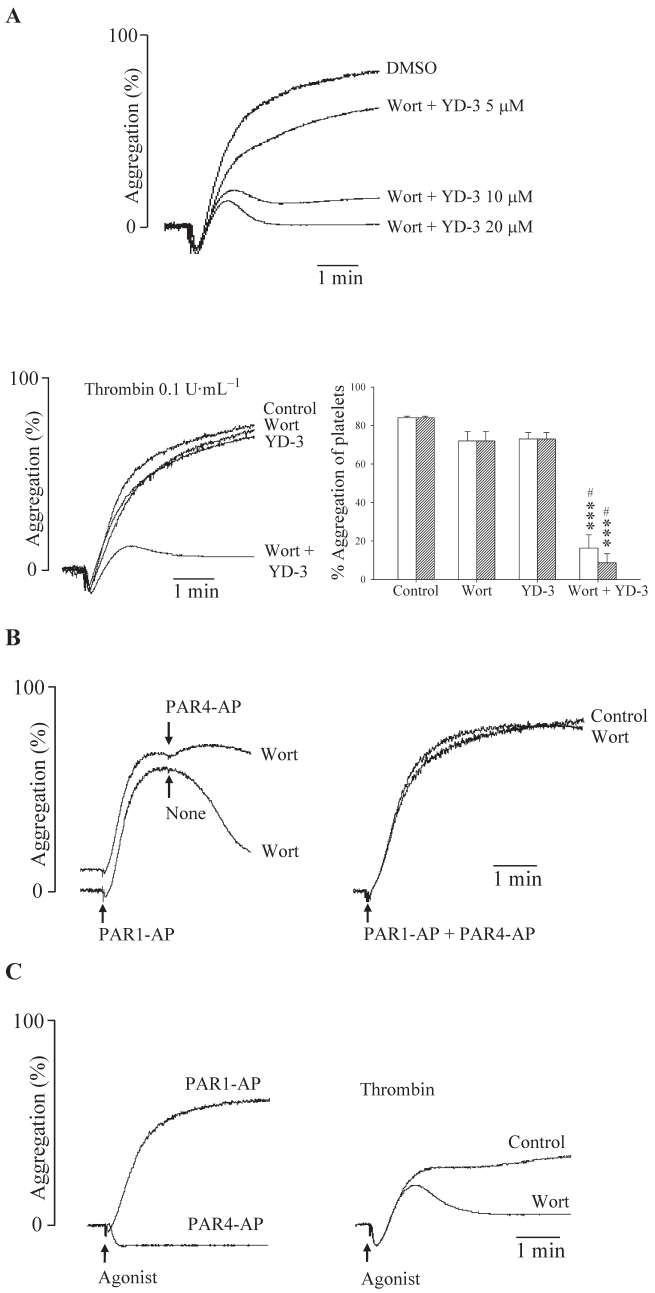

Figure 2

PAR4 is involved in maintaining thrombin-induced platelet aggregation. (A) Washed human platelets were incubated with DMSO (control) or YD-3 (5, 10 and 20 μM in the upper panel, 20 μM in the lower panel) in the presence or absence of wortmannin (Wort, 200 nM) at 37°C for 5 min, then thrombin (0.1 U·mL^{-1}) was added to induce platelet aggregation. Representative platelet aggregation traces of three independent experiments are shown. In the lower panel, the extent of maximal aggregation (open columns) and aggregation after 5 min addition of thrombin (final aggregation, hatched columns) were determined and quantified in the histograms presenting means ± SEM ($n = 3$). ***$P < 0.001$ as compared with respective controls. $^{\#}P < 0.001$ as compared with wortmannin alone group. (B) Platelets were pre-incubated with DMSO (control) or wortmannin, then PAR1-AP (20 μM) or PAR4-AP (100 μM) was added at the indicated time point respectively. Representative tracings of three independent experiments are shown. (C) Left panel, PAR4-desensitized platelets were stimulated with either PAR4-AP (100 μM) or PAR1-AP (20 μM) Right panel, PAR4-desensitized platelets were incubated with DMSO (control) or wortmannin (200 nM) at 37°C for 5 min, then stimulated with thrombin (0.1 U·mL^{-1}). Representative platelet aggregation tracing of three independent experiments are shown. AP, activating peptide; DMSO, dimethyl sulphoxide; PAR, proteinase-activated receptor; YD-3, 1-benzyl-3-(ethoxycarbonylphenyl)-indazole.

Wortmannin plus YD-3 accelerates GPIIb/IIIa inactivation in thrombin-stimulated platelets

Platelet aggregation is dependent on the activation of GPIIb/IIIa and fibrinogen binding, while GPIIb/IIIa inactivation can lead to disaggregation of aggregated platelets. In the present study, we investigated the effect of wortmannin and YD-3 on the dynamics of GPIIb/IIIa exposure in thrombin-stimulated platelets. Platelet GPIIb/IIIa activation was monitored by the binding of FITC-conjugated PAC-1, as this monoclonal antibody only binds to the activated form of GPIIb/IIIa. When PAC-1 was added immediately before platelet stimulation (time 0) with thrombin, the percentage of PAC-1-bound platelets increased from resting levels of 0.4% to 98.0% in thrombin-stimulated platelets (Figure 3). In order to determine the duration of GPIIb/IIIa exposure, PAC-1 was added at different time points following thrombin stimulation. As shown in Figure 3, the percentage of PAC-1-bound platelets at 1, 3 and 6 min was not significantly different from that at time 0, suggesting that thrombin-induced GPIIb/IIIa activation could be sustained for at least 6 min without significant decline. Pretreatment with either wortmannin or YD-3 did not significantly affect PAC-1 binding to thrombin-stimulated platelets. In contrast, co-administration of wortmannin and YD-3 resulted in synergism of the inhibitory effect on thrombin-induced PAC-1 binding. The binding of PAC-1 to platelets treated with wortmannin plus YD-3 was reduced by 26.9% when PAC-1 was added at time 0, and by 64.9% when PAC-1 was added 6 min after thrombin stimulation, indicating that blockade of both PI3K and PAR4 led to acceleration of GPIIb/IIIa inactivation. These results suggest that both PI3K and PAR4 are required for persistent activation of GPIIb/IIIa in thrombin-stimulated platelets.

Wortmannin abolishes thrombin-induced Akt activation in human platelets

Akt is a major downstream effector of PI3K in platelets and is thought to play a role in platelet activation and aggregation (Chen *et al.*, 2004; Woulfe *et al.*, 2004). Activation of Akt is regulated by phosphorylation on two residues, Thr308 and Ser473

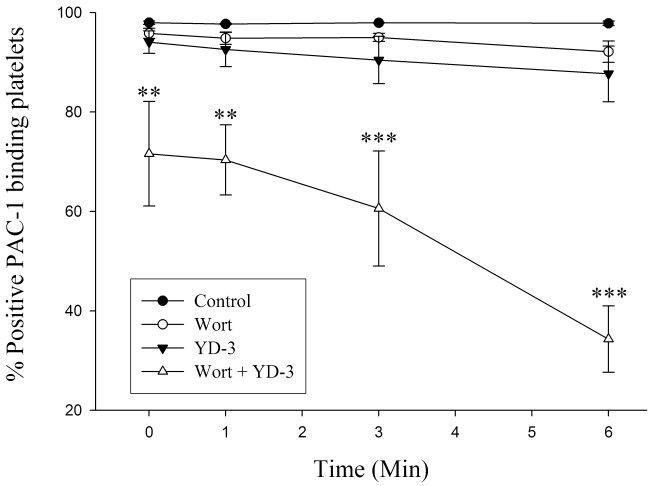

Figure 3

Wortmannin and YD-3 accelerated GPIIb/IIIa inactivation of thrombin-stimulated platelets in a synergistic manner. Washed human platelets were pre-incubated with DMSO, wortmannin (Wort, 200 nM) and/or YD-3 (20 μM) at room temperature for 5 min; FITC-conjugated PAC-1 was then added either immediately before (time 0) or 1, 3 or 6 min after thrombin stimulation (0.1 U·mL^{-1}). The samples were fixed after 20 min with 1% paraformaldehyde. The percentage of PAC-1 positive platelets was analysed by flow cytometry as described in *Methods*. Results are presented as mean ± SEM ($n = 3$). **$P < 0.01$, ***$P < 0.001$ as compared with control. GPIIb/IIIa, glycoprotein IIb/IIIa; YD-3, 1-benzyl-3-(ethoxycarbonylphenyl)-indazole.

(Alessi *et al.*, 1996). Although Akt phosphorylation is predominantly dependent on PI3K, PI3K-independent mechanisms have also been reported. For example, Kroner *et al.* (2000) and Resendiz *et al.* (2007) indicated that thrombin could induce Akt phosphorylation through the PLC pathway in human platelets. Therefore, it is possible that the enhancement of disaggregation by the PAR4 antagonist resulted from inhibition of PI3K-independent Akt phosphorylation. Figure 4 shows that wortmannin alone completely prevented Akt phosphorylation at both Thr308 and Ser473 in thrombin-stimulated platelets, suggesting that thrombin-induced Akt phosphorylation was totally dependent on PI3K in our experimental conditions (Figure 4A). In contrast, YD-3 alone only partly inhibited thrombin-induced Akt phosphorylation and had no effect on the action of wortmannin. As shown in Figure 4B, both PAR1-AP and PAR1-AP induced Akt phosphorylation in human platelets. PAR1-mediated Akt Thr308 phosphorylation occurred rapidly and peaked at 1 min of stimulation and declined thereafter, while PAR4 induced a slower but more sustained response. Furthermore, wortmannin also abolished Akt phosphorylation induced by either PAR1-AP or PAR4-AP (Figure 4B). Therefore, these results indicate that the PAR4 antagonist does not enhance the disaggregatory effect of wortmannin through additional inhibition of Akt phosphorylation.

Effects of wortmannin and YD-3 on thrombin-induced intracellular Ca^{2+} mobilization in human platelets

Previous studies have demonstrated that Ca^{2+} plays a critical role in the activation of GPIIb/IIIa (Sargeant and Sage, 1994). PAR4 is known to contribute to a sustained elevation of intracellular Ca^{2+} in thrombin-stimulated platelets (Covic *et al.*, 2000; Shapiro *et al.*, 2000). However, it is unclear whether PI3K plays a role in regulation of thrombin-induced Ca^{2+} signal (Pasquet *et al.*, 1999; Rosado and Sage, 2000; Voss *et al.*, 2007). We therefore investigated whether the disaggregatory effect of wortmannin and/or YD-3 result from interference with calcium mobilization in platelets. As shown in Figure 5, in the presence of extracellular calcium (1 mM), thrombin elicited a calcium spike followed by a prolonged phase. When platelets were treated with YD-3, the thrombin calcium signal still had a spike-type profile but largely lost the prolonged phase, thus the elevated calcium signal rapidly decayed towards the baseline ($t_{1/2}$ from 72.3 ± 5.7 to 33.3 ± 1.0 s, $P < 0.001$). We found that YD-3 also diminished the ADP-triggered platelet calcium signalling (20–30% inhibition of Ca^{2+} peak as compared with the control); however, it had little or no effect on the decline of the $t_{1/2}$ of $[Ca^{2+}]_i$ and platelet aggregation in ADP-stimulated platelets (data not shown).

In contrast to YD-3, wortmannin did not significantly affect the peak calcium levels or the decrease in the $t_{1/2}$ of $[Ca^{2+}]_i$ in thrombin-stimulated platelets (66.3 ± 4.7 s, $P = 0.40$). Wortmannin was also unable to affect intracellular calcium mobilization in response to either PAR1-AP or PAR4-AP. Further, the combination of wortmannin and YD-3 did not have an additive effect on intracellular calcium mobilization (Figure 5).

Effects of wortmannin and YD-3 on thrombin-induced PKC activation in human platelets

In addition to calcium signalling, agonist-induced PKC activation also contributes to the exposure of GPIIb/IIIa (van Willigen *et al.*, 1996; Hers *et al.*, 1998). In this study, the effects of wortmannin and/or YD-3 on thrombin-induced PKC activation were determined by measuring phosphorylation of MARCKS, which is a major substrate of PKC in human platelets (Elzagallaai *et al.*, 2000). Figure 6A shows that MARCKS phosphorylation in response to

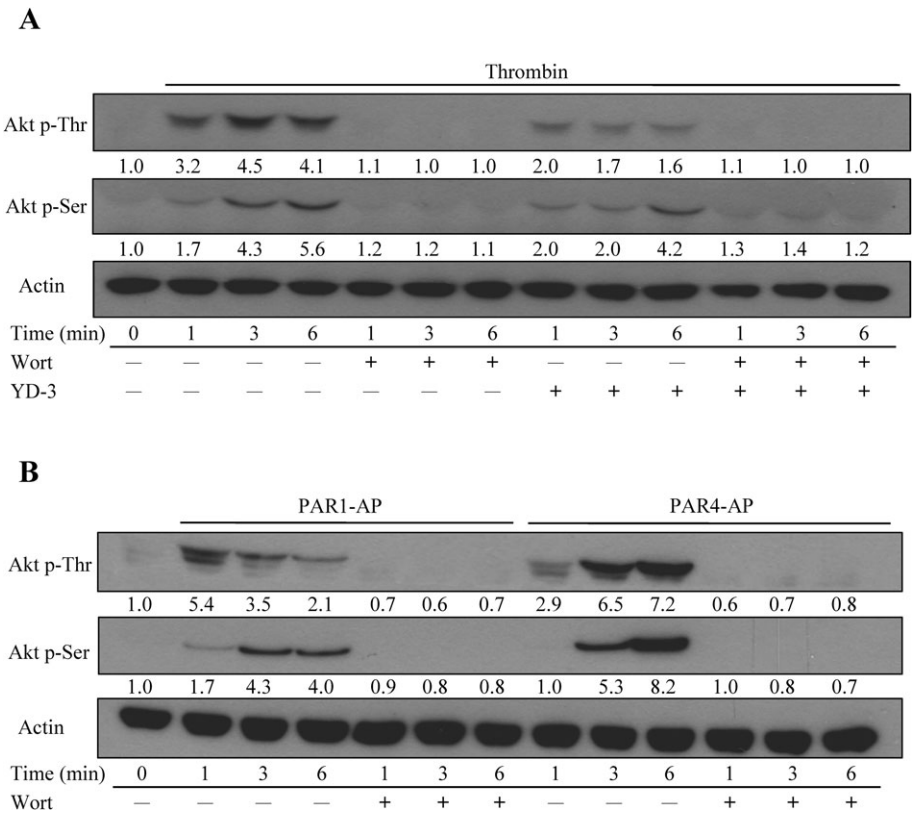

Figure 4

Wortmannin abolished Akt phosphorylation caused by thrombin. Washed human platelets were pre-incubated with DMSO, wortmannin (Wort, 200 nM) and/or YD-3 (20 μM) at 37°C for 5 min. And then, platelets were treated with (A) thrombin (0.1 U·mL^{-1}), (B) PAR1-AP (20 μM) or PAR4-AP (100 μM) for the indicated periods. Platelet lysates were subjected to Western blot analysis for phospho-Akt (Thr308 and Ser473). Similar results were obtained in three separate experiments. AP, activating peptide; PAR, proteinase-activated receptor; YD-3, 1-benzyl-3-(ethoxycarbonylphenyl)-indazole.

thrombin peaked at 1 min, then declined at 6 min. Wortmannin treatment partially inhibited the initial phosphorylation of MARCKS (1 min), but almost completely inhibited the late phase (3–6 min) of phosphorylation induced by thrombin. YD-3 alone only partly inhibited thrombin-induced MARCKS phosphorylation. The combination of wortmannin and YD-3 resulted in complete inhibition of the late activation of PKC, but the early response remained significant, although reduced.

In PAR1-stimulated platelets, MARCKS phosphorylation peaked at 1 min, followed by a gradual decline within 3 min. In contrast, PAR4-AP induced more prolonged MARCKS phosphorylation, which remained detectable for as long as 6 min (Figure 6B). Wortmannin treatment almost completely abolished the late phosphorylation of MARCKS (2–3 min) induced by PAR1-AP without affecting the initial response. In contrast, PAR4-AP-induced MARCKS phosphorylation was more resistant to the action of wortmannin.

In order to determine whether the loss of sustained PKC activation accounts for the ability of wortmannin to reverse platelet aggregation, the phorbol ester TPA was used to directly activate PKC. As shown in Figure 6C, post-addition of TPA (100 nM) to PAR1-stimulated platelets completely attenuated the inhibitory effect of wortmannin. In contrast, TPA only partially prevented the inhibition of thrombin-induced platelet aggregation induced by wortmannin plus YD-3.

To further confirm the importance of sustained PKC activation in irreversible platelet aggregation, we added the general PKC inhibitor GF-109203X immediately after stimulation of platelets with thrombin or APs. Figure 6D shows that post-treatment with GF-109203X did not significantly affect thrombin-induced platelet aggregation; however, when it was combined with YD-3, the aggregation was reduced and became reversible. In contrast, post-treatment with GF-109203X alone was able to reverse platelet aggregation in response to PAR1-AP (Figure 6D) or PAR4-AP (data not shown).

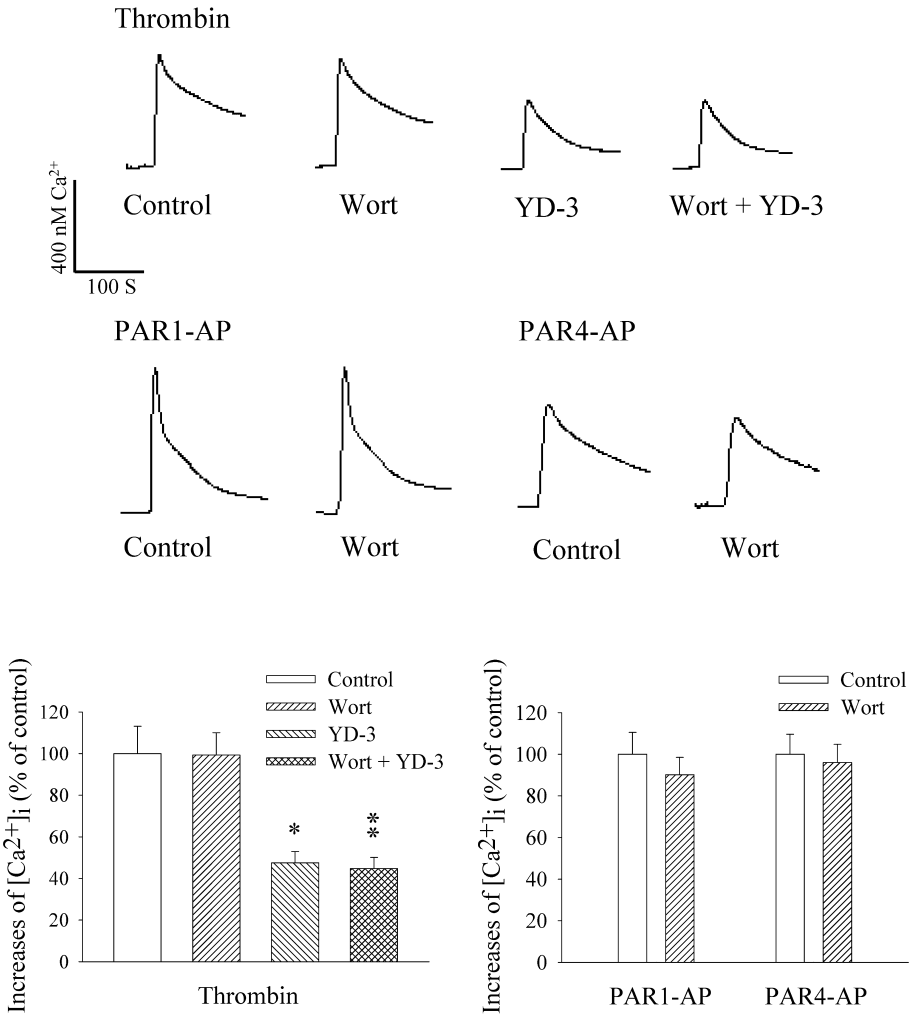

Figure 5

Effects of wortmannin and YD-3 on intracellular calcium mobilization in platelets. Fluo-3-loaded human platelets were incubated with DMSO, wortmannin (Wort, 200 nM) and/or YD-3 (20 μM) at 37°C for 5 min in the presence of 1 mM extracellular Ca^{2+}, then thrombin (0.1 U·mL^{-1}), PAR1-AP (20 μM) or PAR4-AP (100 μM) was added to trigger the increase of $[Ca^{2+}]_i$. Results are representatives of four independent experiments and are quantified in the histograms. Values are mean ± SEM ($n = 4$). *$P < 0.05$, **$P < 0.01$ as compared with control. AP, activating peptide; PAR, proteinase-activated receptor; YD-3, 1-benzyl-3-(ethoxycarbonylphenyl)-indazole.

Akt cannot account for PI3K-mediated PKC activation and irreversible platelet aggregation

We next attempted to determine which signalling molecule is responsible for PI3K-dependent PKC activation and platelet aggregation. The role of Akt, a major downstream effector of PI3K, was investigated by using selective inhibitors (SH-6 and AKT inhibitor V) at concentrations reported to inhibit Akt in human platelets (Resendiz *et al.*, 2007; Yin *et al.*, 2008). As shown in Figure 7A, both SH-6 (30 μM) and AKT inhibitor V (30 μM) significantly decreased the Ser9 phosphorylation of GSK3β induced by thrombin, PAR1-AP and PAR4-AP, which is mainly dependent on Akt in platelets (Li *et al.*, 2008), thus confirming the effectiveness of these two inhibitors. In this condition, however, neither SH-6 nor AKT inhibitor V markedly prevented MARCKS phosphorylation induced by these stimulators (Figure 7B). Moreover, SH-6 and AKT inhibitor V only slightly reduced the maximal extent or the initial rate of platelet aggregation in response to thrombin, PAR1-AP or PAR4-AP, and did not affect the stability of platelet aggregation (Figure 7C). Even in the presence of YD-3, SH-6 or AKT inhibitor V also failed to reverse thrombin-induced platelet aggregation (Figure 7C).

Combined blockade of ADP $P2Y_{12}$ receptor and PAR4 reverses thrombin-induced platelet aggregation

It has been reported that PAR1-mediated PI3K activation is largely dependent on the ADP/$P2Y_{12}$/G_i

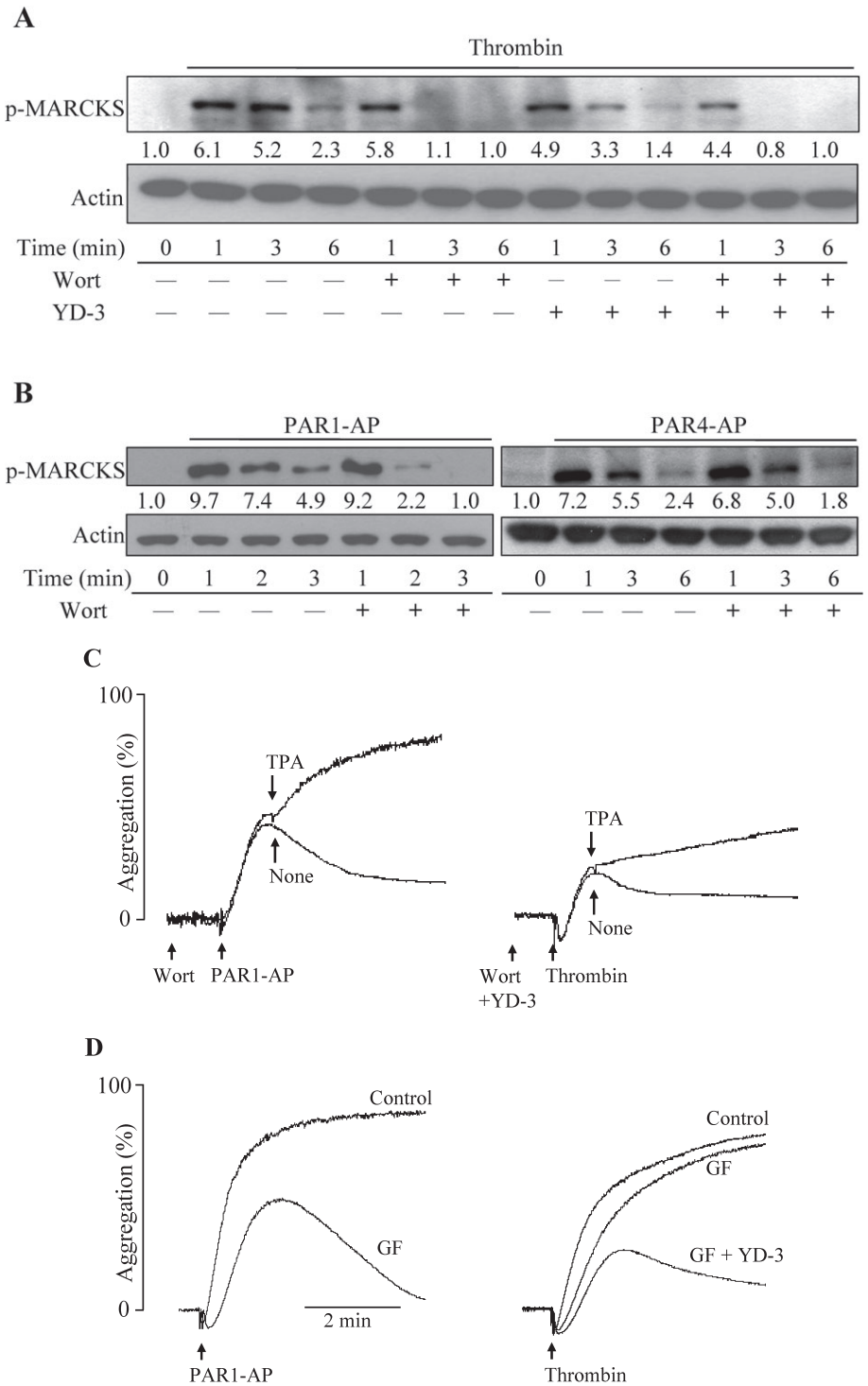

Figure 6

Effects of wortmannin and YD-3 on PKC activation. Washed human platelets were pre-incubated with DMSO, wortmannin (Wort, 200 nM) and/or YD-3 (20 μM) at 37°C for 5 min. And then, platelets were treated with (A) thrombin (0.1 U·mL^{-1}), (B) PAR1-AP (20 μM) or PAR4-AP (100 μM) for the indicated periods. Platelet lysates were subjected to Western blot analysis for phospho-MARCKS. (C) Platelets were pretreated with wortmannin alone or wortmannin plus YD-3, and then PAR1-AP or thrombin was added to trigger platelet aggregation. In some experiments, TPA (100 nM) added as indicated. (D) Platelets were pretreated with DMSO (control) or YD-3, and then PAR1-AP or thrombin was added to trigger platelet aggregation. In some experiments, GF-109203X (GF, 1 μM) was added immediately after stimulation. Similar results were obtained in three separate experiments. AP, activating peptide; MARCKS, myristoylated alanine-rich C kinase substrate; PAR, proteinase-activated receptor; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol 13-acetate; YD-3, 1-benzyl-3-(ethoxycarbonylphenyl)-indazole.

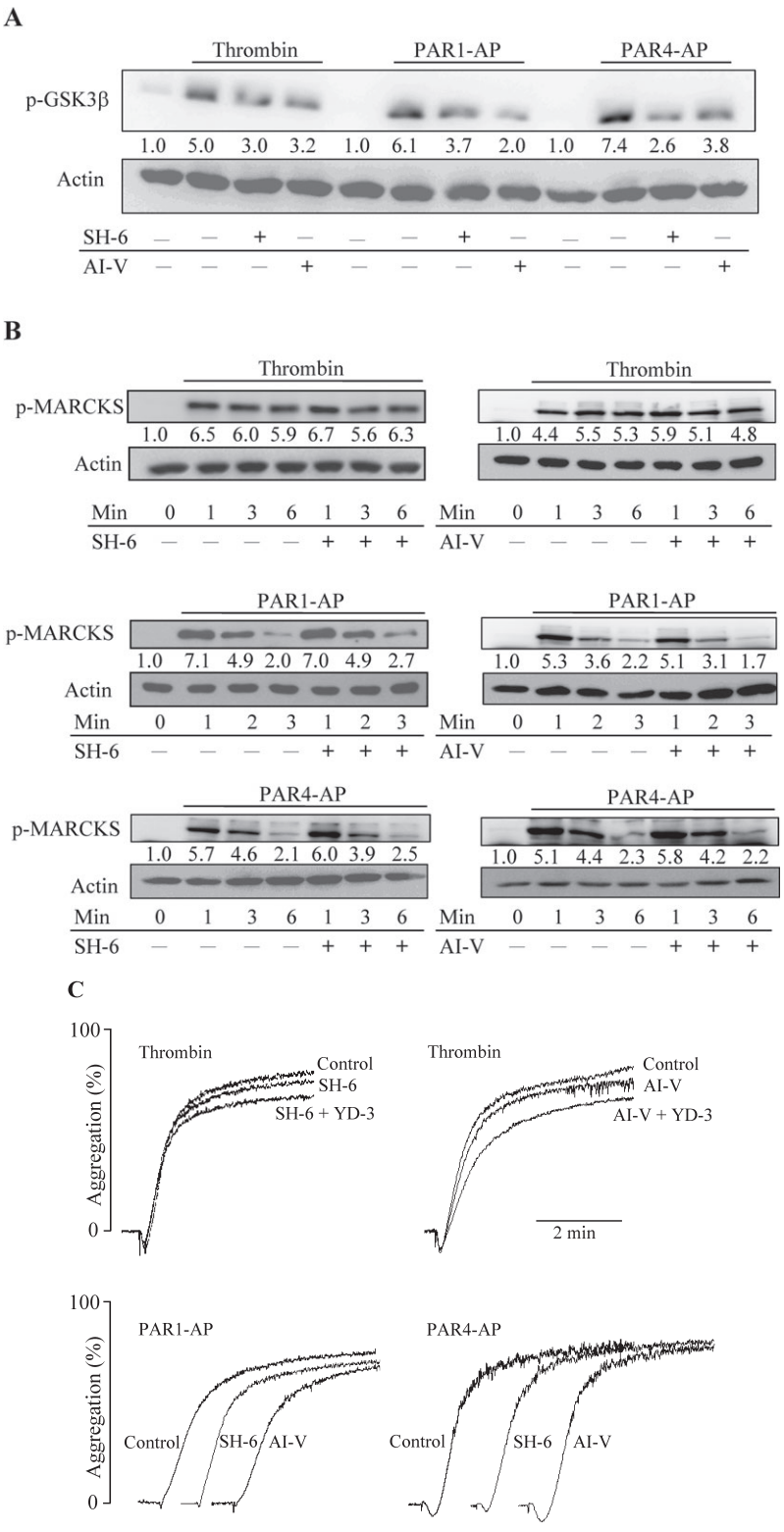

Figure 7

Akt cannot account for PI3K-mediated PKC activation and irreversible aggregation. (A, B) Washed human platelets were pre-incubated with SH-6 (30 μM) or Akt inhibitor V (AI-V, 30 μM) at 37°C for 5 min and 15 min, respectively, and then thrombin (0.1 U·mL^{-1}), PAR1-AP (20 μM) or PAR4-AP (100 μM) was added to stimulate platelets for the indicated periods. Platelet lysates were subjected to Western blot analysis for phospho-GSK3β and phospho-MARCKS. (C) Platelets were pretreated with DMSO, SH-6 or Akt inhibitor V, followed by stimulation with agonists. Results are representatives of three independent experiments. AP, activating peptide; MARCKS, myristoylated alanine-rich C kinase substrate; PAR, proteinase-activated receptor; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C.

pathway (Trumel *et al.*, 1999); we thus investigated whether a $P2Y_{12}$ antagonist is also able to disrupt the stability of thrombin-induced platelet aggregation when in combination with a PAR4 antagonist. As shown in Figure 8A, the $P2Y_{12}$ antagonist 2Me-SAMP (100 μM) abolished Akt phosphorylation, at both Thr308 and Ser473, induced by thrombin or PAR1-AP, and selectively inhibited the late phosphorylation of MARCKS (Figure 8B). In contrast, although 2Me-SAMP also abolished PAR4-AP-induced Akt phosphorylation, MARCKS phosphorylation was less affected than that in PAR_1-stimulated platelets (Figure 8A and B). 2Me-SAMP alone was able to reverse the platelet aggregation induced by PAR1-AP, but not that induced by PAR4-AP or thrombin. As expected, in the presence of both 2Me-SAMP and YD-3, thrombin-induced platelet aggregation was reduced and became reversible (Figure 8C).

Discussion

In the present study, we have demonstrated that in addition to PI3K, PAR4 also contributes to the maintenance of GPIIb/IIIa exposure and platelet aggregation in response to thrombin. Although it has been suggested that PAR4 stabilizes thrombin-induced platelet aggregation (Covic *et al.*, 2002b), there is little direct evidence for such an effect. In this study, several approaches were used to further elucidate the role of PAR4 in this response. First, PAR4 was blocked by using YD-3, which is a selective, non-peptide antagonist of this receptor (Wu *et al.*, 2002; Wu and Teng, 2006; Ofosu *et al.*, 2008). When platelets were cotreated with a PI3K inhibitor and YD-3, thrombin only induced a small wave of platelet aggregation followed by almost complete disaggregation. Second, in PAR4-desensitized platelets, wortmannin was able to reverse platelet aggregation in response to thrombin; the result was the same as that observed in YD-3-treated platelets. Third, PAR4-AP attenuated the inhibitory effect of wortmannin on PAR1-AP-induced irreversible platelet aggregation. Finally, by using PAC-1 binding to determine the duration of GPIIb/IIIa exposure caused by thrombin, we showed that wortmannin plus YD-3 markedly accelerated the inactivation of GPIIb/IIIa in thrombin-stimulated platelets, suggesting that the sustained activation of GPIIb/IIIa, and thus the irreversible aggregation, is dependent on both PAR4 and PI3K.

It has been reported that stimulation of either PAR1 or PAR4 can lead to PI3K activation and Akt phosphorylation in human platelets (Kim *et al.*, 2004; Resendiz *et al.*, 2007). Here, we also showed that PAR1-AP and PAR4-AP can induce PI3K-dependent Akt phosphorylation but with different kinetics. However, inhibition of PI3K with wortmannin resulted in a reversal of the platelet aggregation mediated by PAR1, but not that induced by PAR4, indicating that PI3K has a different role in PAR1-mediated platelet responses than in those induced by PAR4. To investigate the mechanisms underlying this difference, we examined the effects of wortmannin on PKC activation and the increase in intracellular Ca^{2+}, which are the major signalling pathways involved in the induction of platelet aggregation. In PAR1-stimulated platelets, wortmannin selectively inhibited the late phosphorylation of MARCKS; this is consistent with previous findings in which PKC activation was determined by measuring pleckstrin phosphorylation (Toker *et al.*, 1995; Zhang *et al.*, 1995). As wortmannin did not affect the PAR1-mediated Ca^{2+} signalling, it is possible that the late stage of PAR1-induced PKC activation occurs via a PI3K-dependent mechanism rather than through the PLC/DAG/Ca^{2+} pathway. Because PAR1-induced PLC signalling was relatively transient ($t_{1/2}$ of $[Ca^{2+}]_i$ decline was 31.5 ± 4.0 s), the maintenance of GPIIb/IIIa exposure and platelet aggregation may largely rely on PI3K-mediated late PKC activation. Indeed, we observed that post-addition of TPA could attenuate the inhibition of PAR1-induced platelet aggregation produced by wortmannin. In contrast, both PAR4-AP-induced PKC activation and Ca^{2+} mobilization were prolonged and relatively resistant to the effects of wortmannin, indicating that PI3K does not play an important role in PAR4 signalling, and this would also explain why PAR4-AP can induce irreversible platelet aggregation in the absence of PI3K activity. In the case of thrombin-activated platelets, disaggregation only occurred when platelets were treated with both wortmannin and YD-3, suggesting that PI3K-mediated PKC activation and PAR4-mediated signalling, especially the prolonged $[Ca^{2+}]_i$ elevation, are two independent and redundant pathways, activation of either pathway is sufficient to maintain thrombin-induced irreversible platelet aggregation.

Akt is the major downstream target of PI3K. Activated PI3K generates $PI(3,4,5)P_3$ phospholipids, which are necessary for the recruitment of Akt into membranes, and Akt is consequently activated through phosphorylation at Thr308 by phosphoinositide-dependent kinase 1 (PDK-1) (Toker and Newton, 2000). For full activation, Akt requires phosphorylation at Ser473 by a mammalian target of Rap (mTOR) (Hresko and Mueckler, 2005; Sarbassov *et al.*, 2005). Genetic or pharmacological disruption of Akt has been shown to impair platelet secretion and to delay platelet aggregation,

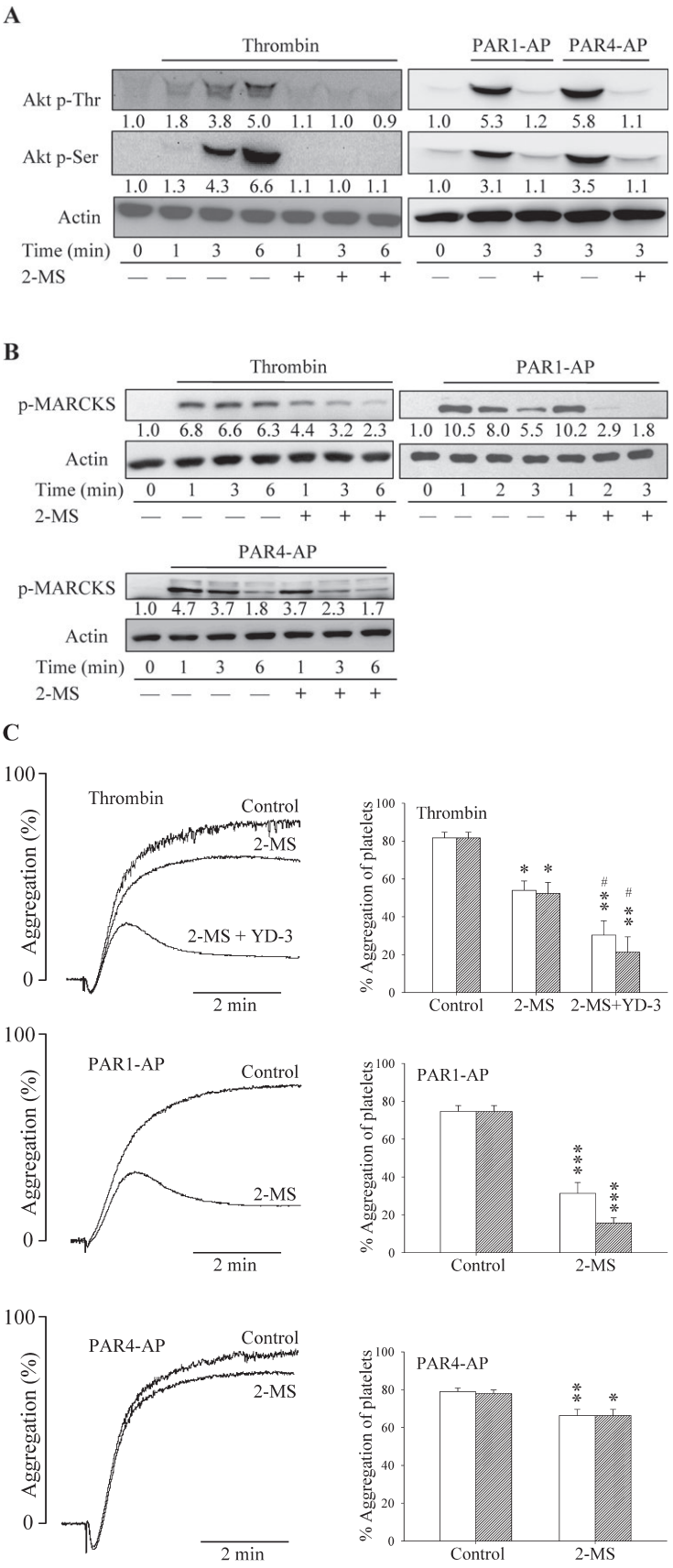
A
Thrombin
PAR1-AP
PAR4-AP
Akt p-Thr
1.0 1.8 3.8 5.0 1.1 1.0 0.9 1.0 5.3 1.2 5.8 1.1
Akt p-Ser
1.0 1.3 4.3 6.6 1.1 1.0 1.1 1.0 3.1 1.1 3.5 1.1
Actin
Time (min) 0 1 3 6 1 3 6 0 3 3 3 3
2-MS — — — — + + + — — + — +
B
Thrombin
PAR1-AP
p-MARCKS
1.0 6.8 6.6 6.3 4.4 3.2 2.3 1.0 10.5 8.0 5.5 10.2 2.9 1.8
Actin
Time (min) 0 1 3 6 1 3 6 0 1 2 3 1 2 3
2-MS — — — — + + + — — — — + + +
PAR4-AP
p-MARCKS
1.0 4.7 3.7 1.8 3.7 2.3 1.7
Actin
Time (min) 0 1 3 6 1 3 6
2-MS — — — — + + +
C
Thrombin
Control
2-MS
2-MS + YD-3
Aggregation (%)
100
0
2 min
% Aggregation of platelets
Control 2-MS 2-MS+YD-3
PAR1-AP
PAR4-AP

Figure 8

Combined blockade of $P2Y_{12}$ receptor and PAR4 reversed thrombin-induced platelet aggregation. Washed human platelets were pretreated with DMSO, YD-3 (20 μM) and/or 2Me-SAMP (2-MS, 100 μM) followed by stimulation with thrombin (0.1 $U \cdot mL^{-1}$), PAR1-AP (20 μM) or PAR4-AP (100 μM) for the indicated periods. Platelet lysates were subjected to Western blot analysis for (A) phospho-Akt (Ser473 and Thr308) or (B) phospho-MARCKS. (C) Platelets were incubated with DMSO, YD-3 and/or 2Me-SAMP at 37°C for 5 min, and agonists were then added to induce platelet aggregation. The extents of maximal aggregation (open columns) and aggregation after 5 min of stimulation (final aggregation, hatched columns) were determined. Results are representatives of three independent experiments and are quantified in the histograms. Values are mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 as compared with respective controls. $^{\#}P$ < 0.05 as compared with 2-MS alone group. 2Me-SAMP, 2-methylthioadenosine 5′-monophosphate triethylammonium salt; AP, activating peptide; MARCKS, myristoylated alanine-rich C kinase substrate; PAR, proteinase-activated receptor; YD-3, 1-benzyl-3-(ethoxycarbonylphenyl)-indazole.

but there are no major defects in the stability of platelet aggregates (Chen *et al.*, 2004; Woulfe *et al.*, 2004; Resendiz *et al.*, 2007). In a very recent study, an Akt inhibitor (Akt inhibitor X) was showed to reverse PAR1-mediated platelet aggregation (Holinstat *et al.*, 2009); however, this must be interpreted with caution as we found that at the concentrations reported, Akt inhibitor X induced platelet activation by itself as judged by platelet shape change (C-C. Wu, unpubl. data). In the present study, we used two structurally different inhibitors of Akt, that is, SH-6 and Akt inhibitor V, to further investigate the relationship between Akt and PI3K-dependent PKC activation. Both Akt inhibitors effectively reduced phosphorylation of the Akt substrate GSK3β with no non-specific effects on platelet activation. Unlike wortmannin, the Akt inhibitors failed to affect PAR1-AP or thrombin-induced PKC activation. Consistent with these data, Akt inhibitors alone or in combination with a PAR4 antagonist also failed to reverse platelet aggregation in response to PAR1-AP or thrombin. These results indicate that in PAR1- or thrombin-stimulated platelets, Akt is not the major regulator of PI3K-dependent PKC activation and cannot account for PI3K-mediated irreversible platelet aggregation. Another potential candidate for this role is PDK-1, which lies between PI3K and Akt. Upon activation of PI3K, PDK-1 translocates to plasma membrane where it activates Akt. In addition to Akt, PDK-1 also regulates many protein kinases such as PKC, PKA and PKG (Belham *et al.*, 1999; Newton, 2003; Mora *et al.*, 2004). It has been shown that the Ca^{2+}-insensitive, novel isoforms of PKC (δ and θ) and the Ca^{2+}/DAG insensitive, atypical forms of PKC (ζ) can be phosphorylated and activated by PDK-1 in a PI3K-dependent manner (Le Good *et al.*, 1998; Villalba *et al.*, 2002; Frey *et al.*, 2006; Taniguchi *et al.*, 2006). We therefore tried to investigate the role of PDK-1 in human platelets by using UCN-01, which is the only commercially available inhibitor of PDK-1 to date. Unfortunately, at concentrations (100 and 200 nM) that abolished Akt Thr308 phosphorylation, UCN-01 reduced the MARCKS phosphorylation and platelet aggregation mediated by both PAR1 and PAR4 (data not shown). One possible explanation for this finding is that, as well as being a PDK-1 inhibitor, UCN-01 also directly inhibits PKC activity (Takahashi *et al.*, 1989); thus it is difficult to distinguish the relationship between these signalling molecules using this method. Nevertheless, we speculate that PDK-1 may play a critical role in mediating PI3K-dependent PKC activation in PAR1-stimulated platelets, but there is a need for more specific inhibitors and strategies to test this hypothesis.

As shown in previous studies and in the present work the activation of PI3K mediated by both PAR1 and PAR4 is largely dependent on stimulation of the $P2Y_{12}/G_i$ pathway by ADP released from platelets (Trumel *et al.*, 1999; Kim *et al.*, 2004). However, only PAR1-mediated late PKC activation was markedly inhibited by the $P2Y_{12}$ antagonist 2Me-SAMP. This result further confirms that the two PAR signals have a different dependence on the $P2Y_{12}$/PI3K pathway. Therefore, $P2Y_{12}$ antagonists should be able to mimic the action of PI3K inhibitors on the stability of platelet aggregates. Indeed, similar to wortmannin, 2Me-SAMP reversed the platelet aggregation mediated by PAR1, but not that mediated by PAR4. Moreover, co-administration of 2Me-SAMP and YD-3 also caused disaggregation in thrombin-stimulated platelets. These findings are of potential clinical significance because platelet aggregation caused by thrombin is refractory to clinically available $P2Y_{12}$ antagonists; co-administration of PAR4 antagonists may thus improve the efficacy of $P2Y_{12}$ antagonists in treating arterial thrombosis. In addition, the selective inhibition of late aggregation in response to thrombin is of potential benefit, because it limits thrombus propagation but spares initial thrombus formation and may thus cause less bleeding complications.

We have proposed a model for the mechanisms of thrombin-induced irreversible platelet aggregation (Figure 9). In this model, thrombin first activates PAR1 and elicits transient Ca^{2+} and PKC signalling, resulting in initial but reversible platelet aggregation. During platelet activation, ADP is released from platelets and then acts on the $P2Y_{12}$ receptor to activate PI3K, which in turn recruits

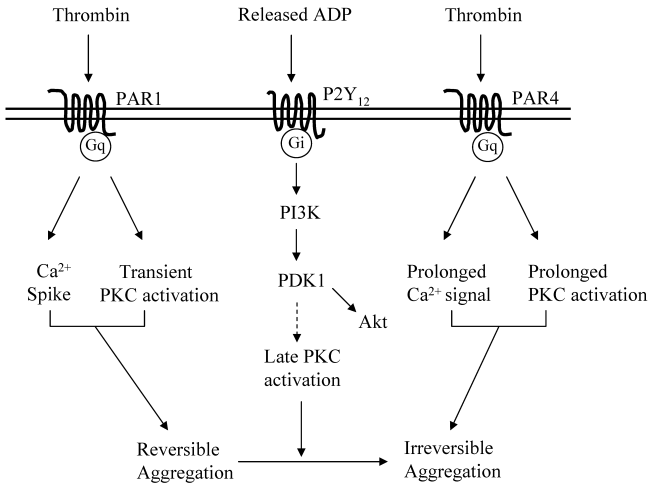

Figure 9

Schematic model for the regulation of stability of platelet aggregation in response to thrombin. PAR1 stimulation by thrombin induces transient Ca^{2+} and PKC signals, which are responsible for eliciting initial but reversible platelet aggregation. During platelet activation, ADP is released from platelets and then acts on the $P2Y_{12}$ receptor to activate PI3K, which turns to recruit PDK-1 and contributes to maintenance of PKC activation. These events help to stabilize and maintain PAR1-mediated platelet aggregation. In addition, PAR4 stimulation by thrombin induces slow but prolonged Ca^{2+} and PKC signals, which are independent of the $P2Y_{12}$/PI3K pathway, and thus provides additional support for maintaining irreversible platelet aggregation in response to thrombin. PAR, proteinase-activated receptor; PDK-1, phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C.

PDK-1 and contributes to maintenance of PAR1-mediated PKC activation. In addition, stimulation of PAR4 by thrombin induces slow but prolonged Ca^{2+} and PKC signals, which are independent of PI3K. Through these mechanisms, PAR4 acts in parallel with the $P2Y_{12}$/PI3K pathway to stabilize platelet aggregates. Inhibition of either pathway does not significantly impair the stability of platelet aggregation as the remaining pathway can compensate. This suggests that co-administration of PAR4 antagonists and $P2Y_{12}$ antagonists may be needed to control thrombin-induced platelet aggregation in arterial thrombotic diseases. Therefore, our results provide new insights into the mechanisms of thrombus stabilization and potential applications for antithrombotic therapy.

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Conflicts of interest

The authors declare no conflicts of interest.

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